PHYSICAL METHODS FOR INACTIVATING SPORES:
A CRITICAL ASSESSMENT

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A significant literature has developed around the application of physical techniques to the inactivation of spores. These include pulsed high electric fields, plasma sterilization, and supercritical fluid extraction, in addition to the more traditional autoclaving technique. Pulsed high electric fields electroporate protective membranes, plasma sterilizers generate very reactive species, and supercritical fluids extract biologically significant compounds. Not all these techniques work as described, however. The cortex interferes with electroporation by showing properties similar to an ion channel, plasma sterilizers are sensitive to the composition of the working gas, and supercritical fluids are acidic in the presence of water. The use of an optimized combination of techniques provides a more efficient kill ratio.

INTRODUCTION

A significant literature has developed around the application of physical techniques to the inactivation of spores. These include pulsed high electric fields,[1] plasma sterilization,[2] and supercritical fluid extraction,[3] in addition to the more traditional autoclaving technique. While many claims have been made on the effectiveness of the technique of interest, the mechanism whereby the spores are inactivated is largely unknown. For this reason, we decided to review the literature for scientific merit. We are reporting what turned out to be an interesting expedition.

BACILLUS ANTHRACIS SPORES

The life cycle of *Bacillus anthracis* includes two biologically significant forms: (1) active vegetative cells, and (2) dormant spores. While the vegetative cells are responsible for the pathogenicity of *Bacillus anthracis*, the dormant spores explain their persistence. The persistence is related to the structure that the spore assembles during sporulation. Surrounding the spore is an exosporium (of as yet unknown function) of variable composition and texture. Underlying the exosporium are several layers of proteinaceous spore coats, an outer spore membrane surrounding the peptidoglycan cortex, a germ cell wall, and an inner forespore membrane that separates
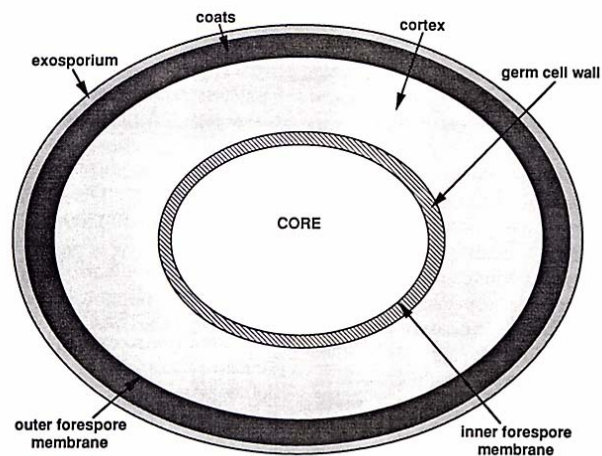

Figure 1: Simplified structure of a spore.

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the cortex from the core (or protoplast). These structures protect the cellular DNA/RNA and enzymes that are stored in the core so that they are able to participate in metabolism initiated during spore germination and outgrowth.[4]

The environmental conditions that lead to spore germination are largely known. Germination begins when spores are exposed to individual germinants (e.g., L-alanine, inosine, proline, asparagine, or a complex between calcium and dipicolinic acid (DPA)), or mixtures of nutrients (e.g., tryptone, yeast extract, or asparagine/glucose/fructose/K^+).[5] After exposure to germinants, a relatively short (5-60 min) incubation period is required.[6] It is believed that the interaction of the germinants with receptors in the inner forespore membrane initiates germination, and leads to permeability changes in the membrane to result in the release of DPA and water uptake.[7] Calcium ion release accompanies the release of DPA, perhaps in combination with the DPA (i.e., as a CaDPA chelate[8,9]), and this release of CaDPA activates lytic enzymes required to degrade the spore's peptidoglycan cortex. While under some conditions calcium ions facilitate spore germination, calcium ions are not needed to germinate spores with a demineralized core (i.e., calcium deficient or H-spores). These demineralized spores can be germinated with a solution of L-alanine and inosine in combination with an electrolyte (e.g., phosphate ions), and are fully viable, yielding normal colonies on agar medium.[10,11,12]

INFLUENCE OF HIGH ELECTRIC FIELDS

Although the release of calcium ions accompanies the germination of wild spores, not much work has been done to investigate the effects of electric fields on germination. Rode and Foster suspended intact *Bacillus megaterium* spores in deionized water, and electrodialized them through a cellophane membrane immersed in a buffered saline solution.[13] Harper, Curran and Pallansch electrodialyzed *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus stearothermophilis* spores with the aid of dialysis tube sacs positioned between two vertical metal plates.[14] Unlike Harper, *et al.*, Rode and Foster observed DPA that Harper, *et al.* attributed to the electrode design and electrolytes used by Rode and Foster that favored germination. Neither group monitored the possible release of calcium ions. When measuring the dielectric properties of *Bacillus cereus* and *Bacillus magaterium* spores, Marquis, et al. concluded that the core contained ionized electrolytes that were tightly bound and extremely immobile within the core.[15,16]

For this reason, we decided to perform several experiments in which an attempt was make to extract calcium ions from wild-type spores of *Bacillus subtilis* using a high electric field. During the course of the experiments, DPA concentrations were also monitored.

Two parallel aluminum plates (6 x 12 inches) were assembled as electrodes. When a suspension of wild-type *Bacillus subtilis* spores in deionized water (5 OD units/mL @ 600 nm, roughly 5×10^8 spores/mL) was introduced
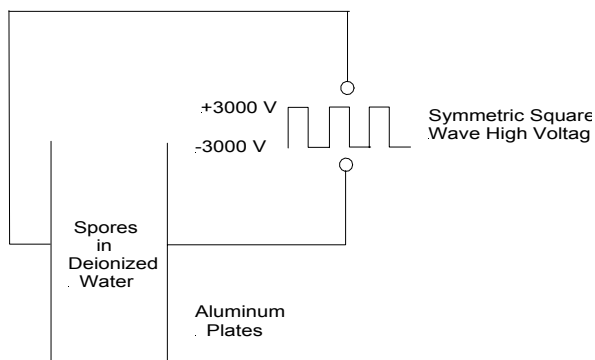
Figure 2: Experimental setup.

between the plates, the spores were exposed to either a low electric field ($\pm$ 15 V incrementally increased to $\pm$ 500 V across the plates), or a high electric ($\pm$ 3000 V across the plates). For the low electric field experiments, the spore suspension was in contact with the electrodes; while for the high electric field experiments, the spore suspension was contained in a FEP Teflon pouch. For both experiments, the spores were "ratcheted" with a dual polarity waveform;[17,18,19,20,21] and for the high field experiments, a field strength of 20 kV/cm was used in each direction. A 20 kV/cm field is adequate to electroporate biological membranes.[22,23,24] After the spores were exposed to the "ratcheting" field for 30 minutes, an aliquot of the suspension was removed, centrifuged, and the supernatant fluid spectrophotometrically analyzed at 270 nm for DPA; and at 600 and 650 nm for calcium after treatment with arsenazo III.[25,26,27,28,29,30,31] When the square-wave potential was reapplied to the remaining suspension, the process was repeated six more times.

The results showed that the supernatant contained 0.5 to 2.3 ppm (gm/gm basis) DPA, and 0.25 to 0.78 ppm (gm/gm basis) calcium. The results were independent of the field strength and the number of times the spores were exposed to the electric field. The values are in the correct ratio for a chelate of CaDPA and are less than a tenth of the maximum concentration [52 ppm (gm/gm basis) for DPA and 8 ppm (gm/gm basis) for Ca] obtained from boiled spores. The results are typical for background levels in biological systems and remained unchanged when the pH of the suspension was reduced to 5.5 to neutralize negative charge in the cortex, or 0.1 millimolar dodecylamine (a chemical germinant when used in higher concentration) at pH 7.4 was added to the suspension to alter the conduction properties of the inner forespore membrane.[32,33,34,35] Microscopic inspections showed that the spores had not germinated.

Given these negative results, efforts were made to better understand the nature of voltage activated ion channels, of which calcium ion channels are a subset.[36] A comprehensive search of the ion channel literature was performed, theoretical modeling of the performance of ion channels (and cortex for reasons noted below) was completed, spores were exposed to chemicals known to antagonize ion channels, and approaches to isolating or "reconstituting" ion channels from the forespore membrane for patch clamp measurements were investigated.[37,38] Some success was achieved in testing antagonists, but the experiments were not carried to a reasonable conclusion. The small size of spores (approx. 0.5 mm) made the isolation or reconstitution of ion channels prohibitively difficult.

The literature search uncovered a variety of issues, some of which have already been described. The most significant, however, is recent work on the EEEE locus within L-type calcium ion channels that actively traps and releases calcium ions in response to voltage/antagonist activity. Adjacent aspartate and glutamate residues provide strategically placed carboxylic groups within the channel to interact with the octahedral geometry of the coordination chemistry for calcium.[39,40,41,42,43,44,45,46] This allows localized binding of calcium within the channel that enhances the properties of the channel as a selective transporter of calcium.[47]

If issues relating to the biochemical composition of the structures are ignored, this idea of localized binding sites within a channel can also be applied to the cortex. The cortex is a porous structure that contains exposed carboxylic groups capable of binding anions, and exposed sugars

and ammonium sites capable of binding cations. Generally assuming the cortex as an array of acidic and basic bonding sites (otherwise known as an acid membrane[48]), it is possible to model the transport of doubly charge DPA anions and doubly charged calcium cations through the cortex and the potential difference that develops as a result. The result is a pair of Nernst equations

$$E_{\left[Ca^{++}\right]_t} = \frac{RT}{F} \Delta \ln\left[Ca^{++}\right]_t$$

$$E_{\left[DPA^{=}\right]_t} = \frac{RT}{F} \Delta \ln\left[DPA^{=}\right]_t$$

(1.1)

where E_i is the Nernst potential, R is the Rydberg gas constant, and F is the Faraday unit of charge. The significance of equation (1.1) is that the ion transport properties of both the cortex and ion channels are characterized by Nernst equations. As a result, it is not possible to differentiate effects relating to ion transport through the cortex from ion transport through the inner forespore membrane. On the other hand, there are also some differences. The energetics associated with the active sites in the cortex are different from that in ion channels, and the cortex and ion channels may be excitable in different ways. Regardless, the cortex could mask the ion transport properties of ion channels from the uninitiated experimentalist. Perhaps this is one reason why spores show apparent resistance to high electric fields.[49] Another reason is that the core is non-conductive and is unable to sufficiently exclude the external electric field required to extract ions through the inner forespore membrane.[50,51]

INFLUENCE OF PLASMA

Having said this, it must be acknowledged that Marquez, *et al.* have reported the ability to kill *Bacillus cereus* spores using 50 kV/cm pulses.[52] Unfortunately, field strengths of this order of magnitude exceed the breakdown potential of air (32 kV/cm).[53] For this reason, van Heesch, *et al.* have proposed that ozone produced by microdischarges inactivated the spores.[54]

There are several ways that ozone can be generated by discharges. For a DC discharge in air, the reactions are

$$e^- + O_2 \xrightarrow{+498.36 \text{ kJ/mol}} e^- + O^{\bullet} + O^{\bullet}$$

$$O^{\bullet} + O_2 + M \rightarrow O_3 + M$$

(1.2)
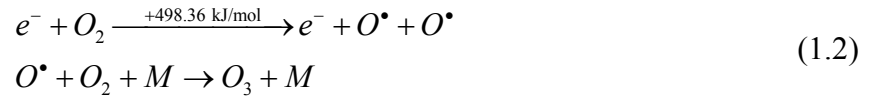
where atomic oxygen reacts with molecular oxygen to form ozone. For a helium glow discharge containing trace (3%) oxygen, the reactions are

$$e^- + He \rightarrow e^- + He*$$

$$He* + O_2 \rightarrow O^{\bullet} + O^{\bullet} + He$$

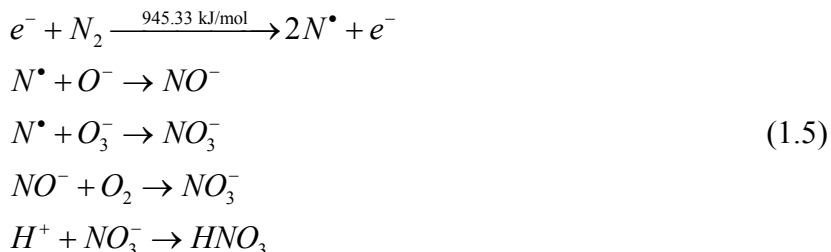$$O^{\bullet} + O_2 + M \rightarrow O_3 + M$$

(1.3)
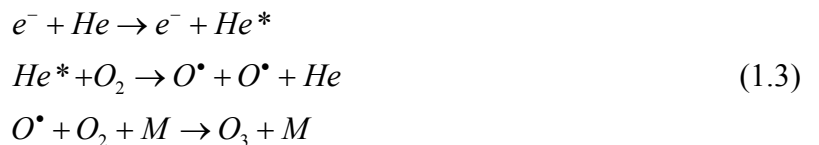
where metastable helium ($He*$) dissociates molecular oxygen into atomic oxygen before the reaction between the two. Finally for photoionization that may accompany near UV irradiation produced by the discharges,

$$h\upsilon + O_2 \rightarrow O^{\bullet} + O^{\bullet}$$

$$O^{\bullet} + O_2 + M \rightarrow O_3 + M$$

(1.4)

where hv is a photon that dissociates the molecular oxygen. Because the discharge conditions that seem to maximize the killing of spores corresponds to reaction (1.3),[2] ozone as a sterilizing agent is implicated. Ozone has been used by a number of research groups in the liquid phase for sterilization purposes.[55,56,57]

On the other hand, the operating conditions of a plasma sterilizer can also be adjusted to maximize the production of nitric acid. The reactions are

$$e^- + N_2 \xrightarrow{945.33 \text{ kJ/mol}} 2N^\bullet + e^-$$
$$N^\bullet + O^- \rightarrow NO^-$$
$$N^\bullet + O_3^- \rightarrow NO_3^- \qquad\qquad (1.5)$$
$$NO^- + O_2 \rightarrow NO_3^-$$
$$H^+ + NO_3^- \rightarrow HNO_3$$

that are particularly efficient in the presence of nitrogen in ambient air under atmospheric pressure conditions. Not much is known about nitric acid as a sterilizing agent except that other acids have been used to kill spores,[58] demineralize spores,[11] extract acid soluble proteins,[59] and lyse cells.[60]

INFLUENCE OF SUPERCRITICAL FLUIDS

The use of supercritical fluids as a substitute for organic solvents as an extractant is now well known.[61] The extraction is accomplished by varying the pressure or temperature of the supercritical fluid until its solubility parameter matches that of the solute. Consequently, if spores are exposed to a supercritical fluid, it might be possible to extract key components of the biological system that are critical for survival. This appears to have been done with maximum efficiency using a microbubble technique.[62]

On the other hand, continued research has shown that the survival rate for microorganisms exposed to supercritical fluids decreases with both the pressure and temperature of the supercritical fluid.[63] An increasing solubility parameter with pressure is normal, but not with increasing temperature. The increasing temperature result leads to the conclusion that the supercritical fluid must be acidifying the aqueous solutions that includes not only the water suspension of microorganisms itself, but also the biological fluids contained within the microorganism. The possibility for such acidification was confirmed when the pH of water in contact with supercritical CO_2 was measured with a pH meter.[64] The pH decreased linearly with CO_2 pressure, suggesting carbonic acid was being formed in the liquid.[65,66,67,68] Once this was established, it was possible to improve yields for the inactivation of spores and the killing of vegetative cells.[69] The improvements were accomplished by increasing the acidity internal to the spore or microorganism, as opposed to the acidity of the external solution.

This latter point is illustrated by the work of Spilimbergo, *et al.* who pretreated their spore suspension with a pulsed electric field (up to 25 kV/cm) before acidification with supercritical CO_2.[70] Without pretreatment, they could not inactivate *Bacillus cereus* spores with 200 bar CO_2 (after 15 and 24 hour exposures). With pretreatment (20 pulses of 25 kV/cm), the inactivation yields increased three orders of magnitude. The most plausible explanation is that

the electric field electroporated the spore membranes. Whether this is true of not, the pretreatment appears to have more efficiently exposed the internal structures of the spore to the supercritical CO_2 that lowered the intracellular pH below the threshold for inactivation.

CONCLUSIONS

The effects of high electric fields, plasmas and supercritical fluids on spores have just been described. Unlike plasmas and supercritical fluids, high electric fields (pulsed or otherwise) do little to inactivate spores. Plasmas seem to inactivate spores by generating reactive species such as ozone or acid. Supercritical fluid CO_2 seems to inactivate spores by making the liquid components of the spore or spore solution more acidic. Without pretreatment with a pulsed high electric field, plasmas are more effective in inactivating spores than supercritical fluids. With pretreatment with a pulsed high electric field, supercritical fluid CO_2 is more efficient in inactivating spores. While more work is needed to fully characterize the biochemistry involved, the evidence suggests that a combination of techniques provides an optimized approach to killing spores.

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